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Review

Metabolomics methods for the synthetic biology of secondary metabolism

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ABSTRACT

Many microbial secondary metabolites are of high biotechnological value for medicine, agriculture, and the food industry. Bacterial genome mining has revealed numerous novel secondary metabolite biosynthetic gene clusters, which encode the potential to synthesize a large diversity of compounds that have never been observed before. The stimulation or "awakening" of this cryptic microbial secondary metabolism has naturally attracted the attention of synthetic microbiologists, who exploit recent advances in DNA sequencing and synthesis to achieve unprecedented control over metabolic pathways. One of the indispensable tools in the synthetic biology toolbox is metabolomics, the global quantification of small biomolecules. This review illustrates the pivotal role of metabolomics for the synthetic microbiology of secondary metabolism, including its crucial role in novel compound discovery in microbes, the examination of side products of engineered metabolic pathways, as well as the identification of major bottlenecks for the overproduction of compounds of interest, especially in combination with metabolic modeling. We conclude by highlighting remaining challenges and recent technological advances that will drive metabolomics towards fulfilling its potential as a cornerstone technology of synthetic microbiology.

1. Introduction

Secondary metabolites constitute an important class of highly valuable compounds covering a broad spectrum of applications, including drugs (e.g. antibiotics, antitumor agents, immunosuppressants), agrochemicals (e.g. pesticides, insecticides, antifeedants), biofuels (e.g. squalene, oleoresin) and food additives (e.g. carotenoids, flavonoids, essential oils). A statistical estimate in 2005 reported approximately 23,000 known bioactive microbial metabolites, of which about 16,500 demonstrated antibiotic activities [1]. However, these compounds are usually produced in very low amounts (or not at all) under typical laboratory conditions in the species from which they originate. Fortunately, recent advances in synthetic microbiology may provide a potential alternative way to access this treasure trove of natural products.

Synthetic biology, which aims to redesign biological systems for novel purposes and applications, enables the transfer of a secondary metabolite biosynthetic pathway from its organism of origin into more amenable heterologous hosts, where the compounds of interest or their precursors can be produced with desired titers [2–7].

One important tool in the synthetic biology toolbox is metabolomics, which catalogues the entire complement of small metabolites in a biological sample [8–11]. General metabolomics applications in synthetic biology have been recently reviewed by Ellis and Goodacre [12], who focused on the integration of metabolomics, fluxomics and metabolic modeling in the design and optimization of engineered microbes.

In this review, we aim to illustrate the role of metabolomics specifically as a research tool in the synthetic biology of secondary metabolism. We first describe the importance of microbial secondary metabolism for synthetic biology. We then discuss the potential of exploiting metabolomics to discover novel compounds and biochemical pathways in microbes. The pivotal role of metabolomics in pathway engineering is further illustrated with examples on the identification of side products and major bottlenecks for the overproduction of compounds of interest. Furthermore, we discuss the potential of metabolomics, integrated with metabolic modeling, as the basis for large-scale synthetic biology projects: metabolomics can be used to supply key information for the improvement of predictive models and contribute to the computer-aided design of synthetic pathways. Finally, we highlight important breakthroughs in analytical methodologies that can support the most recent trends in synthetic microbiology.

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2. Secondary metabolism and synthetic biology

Bacterial genome mining has revealed numerous orphan secondary biosynthetic gene clusters, which potentially encode for novel compounds of high biotechnological value. These gene clusters constitute an almost inexhaustible natural resource of secondary metabolites for synthetic microbiologists [13–16]. The highly modular nature of the biosynthetic machinery responsible for the production of secondary metabolites makes them a particularly attractive target for synthetic biology strategies, both by refactoring gene clusters to produce their product more efficiently [17] and by recombining the modules to increase the fraction of chemical space accessible to biological production systems [18]. This rational engineering approach can further be enhanced by integration with random mutagenesis and metabolic modeling [2,8].

Recent advances in genome synthesis [9,10,19,20] make such strategies realistic on a relatively large scale. An outstanding example of engineering secondary metabolite biosynthesis is the overproduction of the artemisinin precursor artemisinic acid using a synthetic biology approach [21]. Genes encoding for the enzymes participating in consecutive steps in the artemisinin biosynthetic pathways were recruited from *Saccharomyces cerevisiae*, *Artemisia annua*, and *Escherichia coli*, assembled into two operons and transformed into an *E. coli* host strain; subsequently several optimization steps were performed in order to achieve efficient compound production [21,22]. Illustrating the power of a modular synthetic biology strategy, the same isoterpenoid pathway was also engineered towards the biosynthesis of taxadiene, a precursor for the clinically practiced anticancer drug taxol, achieving an increase in titer of approximately 15,000-fold in *E. coli* [23]. Currently, tools for similar biosynthetic engineering of typical secondary metabolite producers such as actinomycetes are also being developed [24].

The development of genome-reduced hosts for heterologous expression of engineered metabolic pathways of interest provides another important component to the synthetic biology toolbox, as it avoids interference from the complex endogenous secondary metabolome. One notable example illustrating the application of genome-minimized hosts for secondary metabolite production is the highly efficient expression of heterologous antibiotics (streptomycin, cephamycin C and pladienolide) and the plant isoterpenoid precursor, amorphadiene, in a genome-minimized strain of *Streptomyces avermitilis* [25].

3. Metabolomics and synthetic microbiology

Metabolomics is the comprehensive analysis of all (or, more realistically: many) metabolites in a biological sample. As metabolomics is the final step in the omics cascade, closest to the phenotype, it provides a direct snapshot of the physiological status of the cell at a certain time point and under specific circumstances [26]. Recent advances in metabolomics studies have been driven by breakthroughs in analytical methodologies in combination with software developments for interpreting experimental data [27]. Extensive research in the field of metabolomics is in turn a driving force for the improvement of the analytical instrumentation, especially in the case of mass spectrometry (MS) [28].

Mass spectrometry has long been a favorite platform for metabolomics studies thanks to its versatility in experimental design (global or targeted analysis, tandem MS for structural information), its high mass accuracy and its high sensitivity to identify and quantify (both relatively and absolutely) very low-abundance metabolites [29]. For microbiological applications, MS is most commonly used in combination with liquid chromatography (LC–MS), with gas chromatography (GC–MS) and capillary electrophoresis (EC–MS) used to a lesser extent [30,31]. All of these methods have been applied successfully for microbiological samples (reviewed in [32]), and the technology is now mature enough for large-scale applications [33]. In the synthetic biology of secondary metabolism, metabolomics can play important roles [34], both as a discovery and a debugging tool (summarized in Fig. 1).

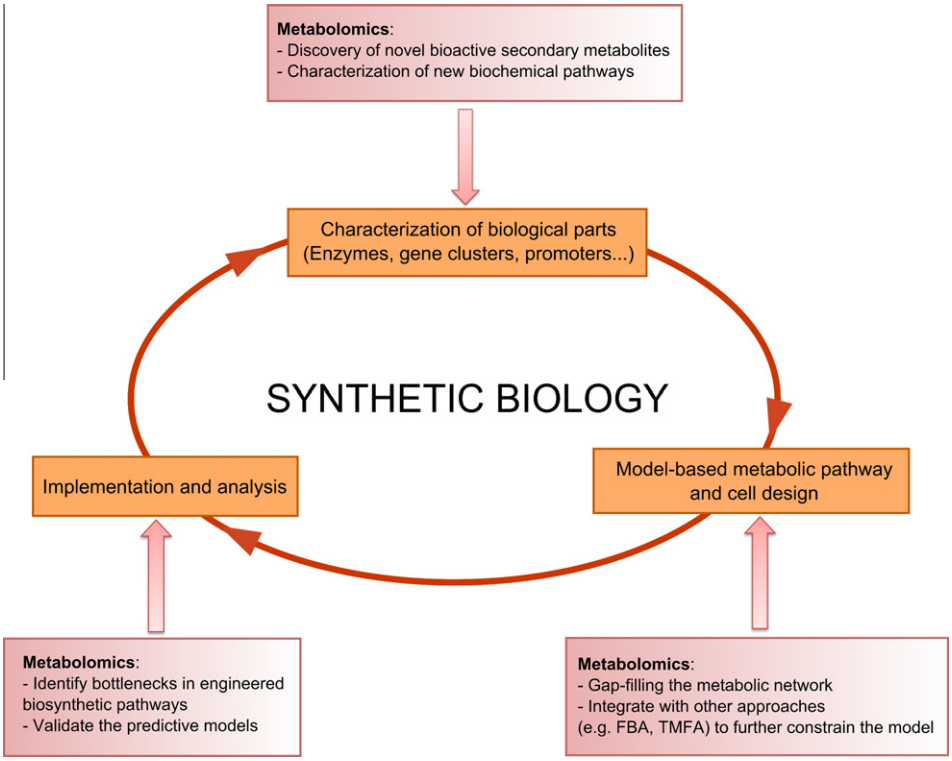

Fig. 1. The roles of metabolomics in the synthetic biology of secondary metabolism.

4. Metabolomics for novel compound discovery in microbes

In its most straightforward application, metabolomics can be used to identify and quantify secondary metabolites, either the products of orphan secondary metabolite gene clusters revealed by genome mining [17,35–37] or the desired products of an engineered strain. The former is particularly promising when applying a comparative metabolomic approach. By comparing the metabolic profile of a wild type strain and its mutants where a silent secondary biosynthetic pathway may be awakened, one can identify novel metabolites that have not yet been characterized, simply by recognizing differentially abundant masses from the two strains' extracts.

The recent identification of a novel bioactive compound synthesized by a type I polyketide synthase (PKS) gene cluster in the congocidine and spiramycin producer *Streptomyces ambofaciens* is a case in point [38]. In this study, overproduction of the metabolite(s) encoded by the cryptic type I PKS in *S. ambofaciens* was achieved by constitutively expressing a putative pathway-specific activator gene (*samR0484*), which encodes a member of the Large ATP-binding LuxR (LAL) family. Comparative metabolic profiling analysis of the methanolic mycelial extracts from the conjugative strain where the cryptic PKS gene cluster was triggered and an empty-vector-containing strain revealed two majors mass species that were detected in the first one but were absent in the second. Further purification by semipreparative HPLC and structure elucidation by proton nuclear magnetic resonance (^{1}H NMR) analysis confirmed that these peaks correspond to four novel 51-membered glycosylated macrolides, named stambomycins A–D. All the four compounds exhibited antiproliferative activities against cancer cell lines and hold potential for antitumor agent development [38].

Detecting novel metabolites of interest by (high- or ultra-performance) LC coupled to (ultra-) high resolution MS followed by structural elucidation by ^{1}H NMR seems to be a powerful general strategy for compound discovery in microbes using metabolomics [39]. Several novel secondary metabolites or new isomers, as well as novel derivatives of known structures, have recently been characterized as products of cryptic biosynthetic gene clusters using this approach; many of these being promising sources of valuable molecules, e.g. bacterial regulators, antimicrobials, siderophores [40–46]. Interestingly, high-throughput approaches for MS identification of novel compounds are now also starting to appear [47].

Furthermore, metabolomics has also served to confirm the proposed products of secondary metabolite gene clusters and to elucidate novel biochemical routes and their regulators by targeted gene disruption and metabolic profiling comparison [48–55]. For example, Pistorius and coworkers examined the involvement of a putative aryl:coenzyme A (CoA) ligase homologue, AuaEII, in the biosynthesis of the quinoline alkaloids aurachins in the myxobacterium *Stigmatella aurantica* Sg a15 [52]. They disrupted the targeted gene *auaEII* and detected the metabolite profiles of this mutant by HPLC coupled to MS and compared to the wild type strain. The inactivation of the target gene was shown to completely abrogate the formation of aurachins, suggesting its indispensable role in the anthranilate loading step of the biosynthetic pathway [34]. Applying this approach, i.e. disruption of targeted genes and analysis of mutant metabolite extracts by LC-MS, the same group has recently deciphered for the first time the complete pathway for aurachin biosynthesis in *S. aurantica* [53] and discovered the new natural product myxoprincomide from *Myxococcus xanthus* [55].

5. Metabolomics to identify the bottlenecks in engineered pathways

Concomitantly to the identification of secondary metabolites by global metabolomics analysis, their side products and key precursors are also detected and quantified. Such comprehensive data can substantially contribute to the examination of metabolic bottlenecks in engineered biosynthetic pathways. Classical metabolic pathway engineering strategies can then be applied to fine-tune the synthetic routes to obtain sufficient product titers [56].

Amongst the most common bottlenecks in engineered biosynthetic pathways are: the depletion of precursors for the desired product; excessive flux towards unwanted side products; poor catalytic capacities of one or several enzyme(s), insufficient to accommodate the high-volume flux to the targeted molecule; and the accumulation of toxic reaction intermediates or the lethality of

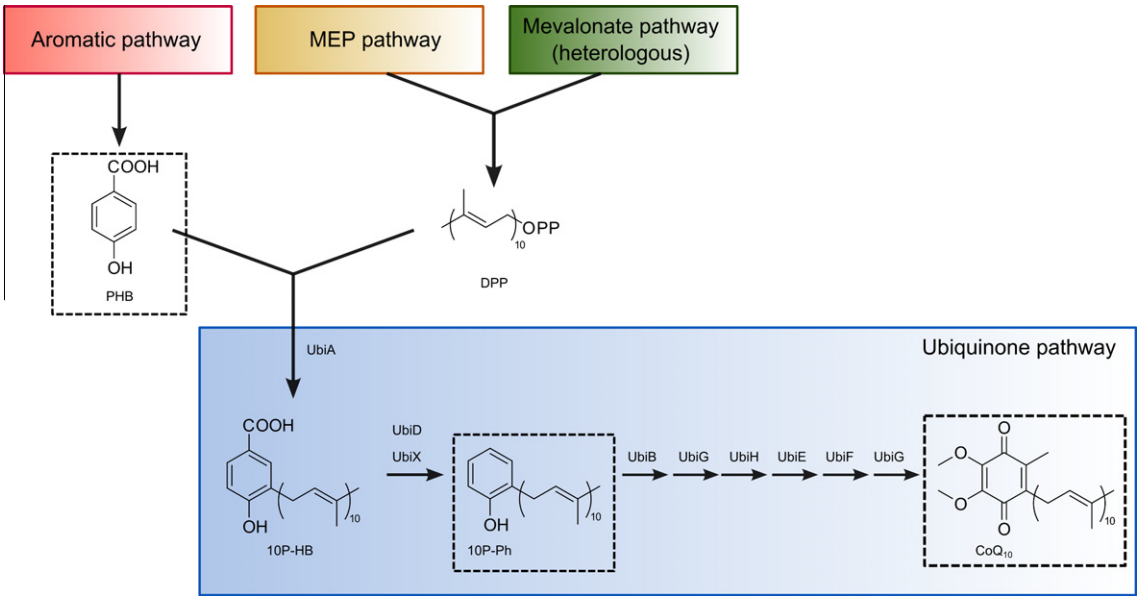

Fig. 2. Scheme showing the steps involved in the engineered *E. coli* for CoQ_{10} overproduction [63]. The metabolites identified by targeted metabolomics are marked with a dashed rectangle. MEP, methylerythritol phosphate; PHB, *para*-hydroxybenzoate; DPP, *E*-decaprenyl diphosphate; 10P-HB, 3-decaprenyl-4-hydroxybenzoate; 10P-Ph, 2-decaprenylphenol; CoQ_{10}, coenzyme Q_{10}.

the product itself, especially at the high titers required for industrial production [57]. By identifying and quantifying side products and/or precursors, metabolomics can guide the synthetic microbiologists to key reactions that require further optimization.

Moreover, as secondary metabolites are formed from building blocks derived from primary metabolism, the supply of the secondary metabolite precursors is usually strongly affected by variations of primary metabolite levels [58]. On the other hand, an imbalance in the secondary metabolic pathway often in turn results in a stress response in central metabolism [59,60]. This implies that to overcome limitations in synthetic pathway function, one needs to expand the focus from the local analysis of the direct biosynthetic route to a metabolomic scale [61,62].

The optimization of the engineered *E. coli* cells for the overproduction of coenzyme Q_{10} (CoQ_{10}) is a good illustration of applying targeted metabolomics for the identification of limitations and subsequent "de-bottlenecking" efforts [63]. In a study performed by Cluis and coworkers, a synthetic pathway for the biosynthesis of CoQ_{10} in *E. coli* was created in a stepwise manner by the deregulated overproduction of two CoQ_{10} precursors: the aromatic acid *para*-hydroxybenzoate (PHB) and the isoprenoid intermediates *E*-decaprenyl diphosphate (DPP) (Fig. 2). To evaluate the efficacy of this complex, multi-branched engineered pathway, targeted metabolomics was applied to quantify the related precursors of each pathway. The secreted PHB and shikimic acid of the aromatic acid pathway were analyzed by optimized HPLC, quinone intermediates were separated from the intracellular extracts by HPLC and identified by tandem MS. In this engineered mutant, an increased content of CoQ_{10} was observed, yet along with a high-level accumulation of 2-decaprenylphenol (10P-Ph), the second intermediate of the ubiquinone pathway. Moreover, the accumulation of the aromatic precursor PHB was also observed, leading to the subsequent optimization by overexpression of UbiA, a PHB decaprenyl transferase involved in the first step in the ubiquinone pathway to enhance the carbon flux towards CoQ_{10}. The resulting mutant showed an increased CoQ_{10} content and a decreased 10P-Ph accumulation. The authors suggested that, as the flux through this pathway is enhanced via the increased production of the precursors (PHB and DPP), one of the pathway enzymes (e.g. UbiB, UbiH, UbiG) may become overloaded, resulting in the accumulation of 10P-Ph. Furthermore, an enhanced activity of UbiA resulted in an improved flux within the ubiquinone pathway, implying the dependence of one or more downstream enzymes on UbiA activity, of which the overexpression will be a key solution for further optimization of the engineered strain [63]. Hence, overproduction of key enzymes, abolishment of competitive pathways or product degradation routes revealed by targeted metabolomics are useful strategies to overcome the limitations in engineered metabolic pathways.

Several other studies applied metabolomics to identify toxicity issues for further optimizing synthetic biological system [60,64]. In one study, the toxic effect of acetic acid (present in pretreated lignocelluloses) on xylose fermentation in engineered *S. cerevisiae* was examined using metabolic profile analysis. This study is also an excellent example illustrating the power of metabolomics as a tool to identify bottlenecks in metabolic engineering for further fine-tuning of synthetic pathways. Time-series metabolomic measurements of intracellular extracts by CE/GC–MS revealed that numerous metabolites involved in the non-oxidative pentose phosphate pathway (PPP) accumulated upon the addition of acetic acid, together with a reduction in xylose consumption. This observation suggested a successful strategy to improve the xylose fermentation yield by targeting PPP-related enzymes, transaldolase or transketolase (encoded by the *TAL1* and *TKL1* genes). The resulting recombinant strain successfully achieved high fermentation levels in the presence of acetic acid and significantly reduced accumulation of PPP intermediates [64].

The application of metabolomics in the "debugging" of engineered microbial strains is not limited to the analysis of the pathway of interest. It is often even more important to characterize unexpected pleiotropic effects on the system. For example, Jankevis et al. [65] have used untargeted metabolomics to characterize the global metabolic rearrangement following induction of a non-coding antisense RNA targeting glutamine synthetase I in *Streptomyces coelicolor*: they could show that this very specific manipulation resulted in a "synthetic metabolic switch" with widespread and rapid changes of metabolite levels. Considering that the natural biosynthesis of secondary metabolites is often the result of stress conditions and accompanied by a metabolic switch (see, e.g. [66]), this is important background information for future attempts at awakening biosynthetic gene clusters. The same holds true for the systematic, unbiased characterization of the metabolomic response to general stress conditions (e.g., salt stress [67]), which are known to induce or favor the overproduction of secondary metabolites in a natural environment.

6. Metabolomics and predictive model building for large-scale synthetic biology projects

One of the crucial steps in constructing artificial biochemical pathways is the identification of a suitable host organism that can accommodate the novel metabolic routes [68]. This task can be facilitated by genome-scale metabolic models and the *in silico* prediction of the phenotype of an engineered organism by a number of constraint-based approaches [69–71]. It is currently relatively straightforward to build the necessary computational models from genome annotations at high-throughput [72], as evidenced by the recent comparative modeling of 37 actinomycete species [73]. However, for application in synthetic biology, the models need to be manually curated in detail. In this process, metabolomics has the potential to play an important role by providing information for model validation and refinement.

The integration of experimentally measured metabolic profiles can reveal targets for gap-filling the metabolic network of model organisms. In the newly sequenced model organism *Chlamydomonas reinhardtii* [74], metabolomics analysis identified 57 metabolites that cannot be generated by the draft metabolic network constructed from genome annotation, suggesting missing reactions or alternative routes to be further elucidated. In a parallel line of validation, additional omics datasets can be integrated to further improve the model quality. For example, it has been shown in the antibiotic-producing *S. coelicolor* that a genome-scale computational model of metabolism correctly predicts fluxes during the metabolic switch to secondary metabolite production, and that these fluxes correlate strongly with observed changes in gene transcript levels [75]. Discrepancies between predicted flux and transcript level lead to the successful identification and correction of modeling errors [75]. The same strategy was applied for the detailed analysis of an industrial antibiotic overproducer, *Streptomyces clavuligerus*, to characterize the changes in the metabolism that distinguish the overproducer from a wild type strain [76]. Together with additional genomic information, these data on a naturally evolved strain (created by random mutagenesis) can suggest important new engineering strategies for a directed creation of overproducers of secondary metabolites of interest [2].

Moreover, metabolomics aids the validation and refinement of genome-scale models via direct measurement of metabolic fluxes, e.g. by feeding microbes with isotopically labeled substrates and quantifying the time-resolved distribution of labeled metabolites by MS and/or NMR [77–81]. For instance, to elucidate the catabolic

and anabolic strategies of the facultative methylotroph *Methylobacterium extorquens* AM1 growing on acetate as a sole carbon source, Schneider et al. [82] applied time-series ^{13}C-labeling targeted metabolomics to unveil the fate of CoA thioesters as key intermediates, in combination with ^{13}C steady-state metabolic flux analysis and proteomics study. The flux calculations were conducted based on a modified genome-scale metabolic network recently constructed by the same group [83]. The isotope-labeling experiment showed that acetate was incorporated by both the ethylmalonyl-CoA (EMC) pathway and the citric acid (TCA) cycle. The majority of acetyl-CoA (68%) was found to enter the TCA cycle and to be oxidized completely to CO_2. A smaller part (21%) was converted into glyoxylate and succinyl-CoA via the EMC pathway while the remaining 5% of acetyl-CoA was condensed with glyoxylate to produce malate. Consequently, the EMC pathway was confirmed to be a functional alternative to the glyoxylate cycle for the assimilation during growth on acetate, as previously shown for the methylotrophic growth in this organism [84], and it also provided glyoxylate for glycine and serine biosynthesis. In addition, the common pathways utilized during growth of *M. extorquens* AM1 on methanol [83,84] and acetate were observed, but with a modification in the connectivity of the metabolic network and a redirected flux towards quick adaptation to the newly supplied carbon source. This study is a nice illustration of applying ^{13}C metabolomics to investigate the metabolic network topology of the central carbon metabolism for consolidating the genome-scale model.

While the first generation of genome-scale metabolic models was using only stoichiometric information to constrain the possible metabolic fluxes, the more recently introduced thermodynamics-based metabolic flux analysis (TMFA) additionally exploits linear thermodynamic constraints based on the calculated Gibbs free energy change for each reaction and the thermodynamic activity of each metabolite in the system [70]. As a consequence, these models are not only able to predict metabolic fluxes with improved accuracy, but are also able to incorporate steady-state metabolite levels – a feature that traditional constraint-based models lack. For example, TMFA combined with NMR-based metabolome analysis resulted in the prediction of thermodynamically feasible flux distributions in the solventogenic strain *Clostridium acetobutylicum* [85]. This methodology, which excludes flux patterns that would be stoichiometrically feasible but thermodynamically impossible, can now be applied to engineer strains for the improved production of various biofuels.

7. Future perspectives

Despite the recent flourish in methodology and proof-of-concept publications and the increasing realization of its potential for supporting synthetic biology research, microbial metabolomics still presents a number of challenges, including both technological issues and limitations of data interpretation [32,86].

Currently, the utilization of complementary separation platforms for LC-MS based metabolomics enables coverage across a large heterogeneous chemical landscape [87,88]. However, metabolite identification by MS remains a rate-limiting step in the metabolomics workflow, especially for the elucidation of novel structures with little prior knowledge, due to the enormous chemical and structural diversity for each detected mass [89]. Thus far, mass identification is mainly based on matching the detected mass with available mass databases and/or on comparing the retention time and mass spectra with authentic standard compounds [90]. There is currently no freely available retention time repository for metabolites, most probably due to the substantial variability in experimental setups (and consequently retention times) among laboratories [91]. Moreover, in contrast to proteomics, efficient algorithms that can reasonably successfully predict and compare the mass fragmentation patterns for tandem MS spectra of metabolites (e.g. MetFrag [92]) still need to be further developed. Efforts to develop such libraries and (semi-) automated tools for data collection from databases (e.g. MassBank [93], Metlin [94], HMDB [95], PubChem [96]) and data annotation therefore should be a priority in the near future.

Most recent novel active metabolite structures, either natural or generated by artificial pathways, have been identified by LC-MS-based metabolomics and elucidated by NMR analysis. One of the most common challenges in NMR-based structure clarification is its inherent low sensitivity: whilst an amount of only a few micrograms is more than sufficient for formula determination by MS analysis, it is hardly enough for structure determination by NMR, despite recent revolutionary improvements that have brought NMR spectroscopy techniques a sensitivity at the nanomole-scale [97]. On the other hand, unfractionated (or "dirty") microbial extracts usually contain complex mixtures of components, which often lead to the NMR spectra that are difficult to interpret due to significant peak overlap. This is an area where synthetic biology will be able to pay back its debt to metabolomics: once it becomes possible to awaken novel secondary metabolite gene clusters by refactoring and produce the end product at high levels and perhaps even in dedicated organelles [7,18], it will be much easier to purify sufficient amounts for rapid NRM-based structure elucidation.

The engineering approach of synthetic biology requires a level of precise control that is rarely achieved in biological systems (see also [98]). The recent blending of synthetic biology with microfluidic technology has attracted attention as one strategy towards advances in this direction, due to its ability to monitor and manipulate systems at the single cell level in an automatic, high-throughput and micro-environmentally controllable fashion [99,100]. To support this development, updates to the metabolomics toolbox are also required. First steps towards microfluidics-based metabolic profiling techniques have already been made (reviewed in [101]), and recently strategies for single cell metabolomics analysis has also been reported [102]. Key remaining challenges include the development of highly sensitive detection techniques, the broad spectrum of required metabolite identification capability and the feasibility to integrate metabolic analytics with the microfluidic devices.

The robust methodologies of advanced analytical chemistry and the technological breakthroughs discussed above, in combination with the ongoing development of new computational approaches for data interpretation, will contribute to bolstering the role of metabolomics as the most powerful ally of synthetic biology.

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